REGULATION OF AMYLOID PRECURSOR PROTEIN EXPRESSION BY MODIFICATION OF ABC TRANSPORTER EXPRESSION OR ACTIVITY

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates generally to the field of amyloid precursor protein and its cleavage to yield β-amyloid, and more specifically to the treatment and prevention of neurological disease, such as Alzheimer's disease, characterized by β-amyloid deposition in tissues.

Description of the Related Art

Alzheimer's disease is a neurodegenerative disorder whose pathological hallmarks include neurofibrillary tangles, senile plaques, and neuronal death. The neurofibrillary tangles contain paired helical filaments composed of hyperphosphorylated tau, while the senile plaques are comprised of an array of proteins deposited around a core of insoluble Aβ peptide (Cummings, J. L. et al., *Neurology 51*:S2-17 (1998)). The cause of neuronal death remains unknown but a considerable body of evidence suggests that it is secondary to an increase in the brain Aβ load (Selkoe, D. J., *Nature 399*:A23-31 (1999)). In relatively rare cases of familial Alzheimer's disease, mutations in any one of 3 genes (amyloid precursor protein (APP), presenilin 1, and presenilin 2) result in early-onset Alzheimer's disease accompanied by increased extracellular levels of the longer isoform of Aβ known as $A\beta_{1-42}$ (Hardy, J. et al., *Nature Neurosci. 1*:355-358 (1999)). Moreover, transgenic animals expressing these gene mutations recapitulate many of the features of AD, including amyloid plaques, cerebrovascular amyloid angiopathy, and neuronal cell death (Price, D. L. et al., *Science 282*:1079-1083 (1998)). Thus, there is a need in the art for compositions and methods that can modulate the synthesis and degradation of β-amyloid, as treatment for or prevention of Alzheimer's disease. Because β-amyloid synthesis is tightly linked to the level of APP in cells, strategies aimed at regulating APP levels are useful as treatment for or prevention of Alzheimer's disease.

BRIEF SUMMARY OF THE INVENTION

In one embodiment, the invention provides a method of regulating expression of APP by regulating expression of an ABC transporter.

In a further embodiment, the ABC transporter is ABCB9.

In a yet further embodiment, the ABC transporter is ABCG4.

In yet a further embodiment, the ABC transporter is ABCG1.

The invention provides a method of regulating expression of APP by contacting an ABC transporter with an antisense oligonucleotide or ribozyme capable of specifically hybridizing to a polynucleotide encoding a human ABC transporter.

In a specific embodiment, the antisense oligonucleotide or ribozyme is capable of specifically hybridizing to a polynucleotide encoding ABCB9.

In another specific embodiment, the antisense oligonucleotide or ribozyme is capable of specifically hybridizing to a polynucleotide encoding ABCG4.

In a specific embodiment, the antisense oligonucleotide or ribozyme is capable of specifically hybridizing to a polynucleotide encoding ABCG1.

The invention further provides targets for developing modulating agents capable of modulating the expression of APP in a cell. In one embodiment, the modulating agents interfere with the expression of an ABC transporter in the cell.

The present invention further provides diagnostic and therapeutic tools for diseases related to the release of amyloid-β protein from the cell membrane, including Alzheimer's disease.

The invention further provides antibodies, such as monoclonal or polyclonal antibodies, that specifically bind ABC transporter proteins so as to interfere with the ABC transporter-regulated expression of APP. Such antibodies can be incorporated into pharmaceutical compositions, which optionally include pharmaceutically acceptable carriers.

In another aspect, the present invention provides a method for detecting the presence of ABC transporter activity in a biological sample by contacting the biological sample with an agent capable of detecting at least one activity including the expression of APP in the biological sample.

In one embodiment, the methods of the present invention are used to treat a subject having a disorder characterized by aberrant or unwanted ABC transporter protein or nucleic acid expression or activity by administering an agent that is an ABC transporter modulator to the subject. In one embodiment, the ABC transporter modulator is an ABC transporter nucleic acid molecule such as an antisense oligonucleotide. In another embodiment, the ABC transporter modulator is a polypeptide antibody (or fragment thereof), peptide, peptidomimetic, or other small molecule, *e.g.* a molecule that is carbohydrate-based, lipid-based, nucleic acid-based, natural organic-based, or synthetically derived organic-based, wherein administration of the agent results in a decrease in APP expression, and ultimately, amyloid-β protein production.

The present invention also provides a diagnostic assay for identifying the presence or absence of a genetic alteration characterized by at least one of (i) aberrant modification or mutation of a gene encoding an ABC transporter protein; (ii) mis-regulation of the gene; and (iii) aberrant post-translational modification of an ABC transporter protein, wherein a wild-type form of the gene encodes a protein with an ABC transporter activity; wherein the genetic alteration results in an alteration in intracellular APP expression levels.

In another aspect the invention provides a method for identifying a compound that binds to or modulates the activity of an ABC transporter protein, by providing an indicator composition comprising an ABC transporter protein having ABC transporter activity, contacting the indicator composition with a test compound, and determining the effect of the test compound on ABC transporter activity in the indicator composition to identify a compound that modulates the activity of an ABC transporter protein, as indicated by an alteration in APP expression levels.

A further aspect of the invention relates to an animal model of disease characterized by β-amyloid plaque deposition, wherein brain cells of an animal are transfected with a construct encoding an ABC transporter, such that the cells exhibit an increase in APP expression.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram indicating the cleavage sites and membrane orientation of APP, resulting in the production of $A\beta_{1-40}$ and $A\beta_{1-42}$.

BRIEF DESCRIPTION OF THE SEQUENCE IDENTIFIERS

SEQ ID NO:1 discloses the polynucleotide sequence for the ABC transporter, ABCB9.

SEQ ID NO:2 discloses the polynucleotide sequence for the ABC transporter, ABCB1.

SEQ ID NO:3 discloses the polynucleotide sequence for the ABC transporter, ABCA2.

SEQ ID NO:4 discloses the polynucleotide sequence for the ABC transporter, ABCG4.

SEQ ID NO:5 discloses the polynucleotide sequence for the ABC transporter, ABCG1.

SEQ ID NO:6 discloses the amino acid sequence for the ABC transporter, ABCB9.

SEQ ID NO:7 discloses the amino acid sequence for the ABC transporter, ABCB1.

SEQ ID NO:8 discloses the amino acid sequence for the ABC transporter, ABCA2.

SEQ ID NO:9 discloses the amino acid sequence for the ABC transporter, ABCG4.

SEQ ID NO:10 discloses the amino acid sequence for the ABC transporter, ABCG1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based, at least in part, on the discovery that expression of ABC transporters, ABCB9, ABCG4 and ABCG1, are involved in modulating the expression levels of amyloid precursor protein (APP). ABC transporter molecules are transmembrane proteins that catalyze ATP-dependent transport of endogenous or exogenous substrates across biological membranes. ABC transporters have been associated with the transport of polypeptides, *e.g.,* a neurotoxic polypeptide, such as β-amyloid, which is involved in Alzheimer's disease. Other neurological diseases caused by neurotoxic polypeptides include prion diseases, Parkinson's disease, Huntington's disease, etc. (*see* Hardy et al., *Science 282*:1075-1079 (1998)). In particular, ABC transporters are associated with the transport of substrates across the blood-brain barrier. In addition, ABC transporters are associated with multidrug resistance found in cells, such as cells that are refractory to cytotoxic anti-cancer drugs (Borst, P., *Sem. Cancer Bio. 8*:131-134 (1997)).

It is well established that Aβ is constitutively produced by sequential endoproteolytic cleavage of APP by enzymes termed β- and γ-secretase (Selkoe, D. J., *Nature 399*:A23-31, 1999). (Figure 1).

Although about 90% of the Aβ normally released from cells is $A\beta_{40}$, the $A\beta_{42}$ form appears to contribute more to the earliest deposition of amyloid during development of Alzheimer's disease. $A\beta_{42}$ remains the predominant constituent of most amyloid plaques as the disease progresses.

Rogers et al. (*J. Biol. Chem. 274*:6421-6431 (1999)) showed that IL-1 substantially induced APP synthesis in primary human astrocytes and astrocytoma cells by increasing message translation. IL-1 enhanced the translational efficiency of astrocytic APP mRNA. Induced APP synthesis was not observable until after 6 hours of IL-1β stimulation in both primary astrocytes and in U373MG astrocytoma cells. These data suggest that enhanced message translation in response to IL-1 in astrocytes is responsible for increased APP synthesis. It is of interest to identify other regulators of APP expression.

Selected members of the ATP-binding cassette (ABC) superfamily of transporters are responsible for the energy-dependent efflux of a variety of lipophilic and amphipathic

molecules from cells (Van Veen, H. W. and Konings, W. N., *Biochim. Biophysica. Acta. 1365*:31-36, 1998; Croop, J. M., *Methods Enzymol. 292*:101-116, 1998; Kuchler, K. and Thorner, J., *Endocrine Rev 13*:499-514 (1992); Ambudkar, S. V. et al., *Annu. Rev. Pharmacol. Toxicol. 39*:361-398 (1999); Yakushi, T. et al., *Nature Cell Biol. 2*:212-218 (2000)).

1. Regulation of APP Expression by ABC Transporter Expression

The present invention shows that APP expression in cells can also be regulated by regulating the expression of ABC transporter, such as ABCB9, ABCG4 and ABCG1, in the cells. As discussed in detail in the Example, cells in culture were transfected with a variety of constructs in order to investigate the effect of ABC transporter expression on APP expression. In one set of experiments, WT6 cells (293-EBNA cells stably transfected with a wild-type APP construct) were transfected with a construct encoding β-galactosidase or one of two ABCB9 mutant proteins. These cells all served as controls. A fourth population of WT6 cells was transfected with a construct encoding ABCB9. 48 hours after transfection, cells were incubated for 4 hours, then cellular APP was assayed by a Western blot analysis using an antibody capable of recognizing the N-terminus of APP.

The WT6 cells transfected with the ABCB9 construct exhibited APP expression levels twice that of the cells transfected with the β-Gal construct or either of the two mutant controls. These results indicate that expression of APP is regulated by or in concert with expression of ABC transporter protein ABCB9.

In a second series of experiments, 293-EBNA cells (HEK 293 cells overexpressing the Epstein Barr Nuclear Antigen) were used. The transfection with ABCB9 and the controls was performed as described above for the WT6 cells. The cells transfected with ABCB9 exhibited APP expression levels of 130% that of the control level. APP expression levels in the cells transfected with the two mutant-encoding constructs did not differ significantly from the β-Gal control.

The results of these experiments indicate that APP expression is upregulated when expression of an ABC transporter protein is increased. As the promoter differed in the two cells types (CMV promoter in WT6 cells; native APP promoter in 293-EBNA cells) the increased APP synthesis relates to APP expression itself, and is not an artifact of the system or

promoter used in the experiments. The invention therefore relates to the up and down regulation of APP expression by increasing or decreasing expression of ABCB9 gene.

Similar results were obtained from transient transfection studies of WT6 cells and SM4 cells (293 EBNA cells stably transfected with a construct encoding a Swedish mutant APP-695 protein) with a construct encoding β-glactosidase, wild type ABCG4, or one of three ABCG4 mutants. In both types of cells, while APP levels of cells transfected with each of the three constructs encoding mutant ABCG4 proteins were similar to those of cells transfected with a construct encoding β-galactosidase, transfection with a construct encoding wild type ABCG4 protein increased the APP expression level by 40% compared to transfection with a construct encoding β-galactosidase.

Similar results as those obtained for ABCB9 or ABCG4 were obtained when the ABC transporter employed was ABCG1.

Although the results herein are based on use of ABCB9, ABCG4 or ABCG1, the invention is not limited to these three ABC transporters. The invention encompasses all ABC transporters expressed in brain tissue, including but not limited to ABCA1, ABCA2, ABCA3, ABCA5, ABCA6, ABCA8, ABCA9, ABCB1, ABCB6, ABCB9, ABCC5, ABCC10, ABCD1, ABCD2, ABCD3, ABCD4, and ABCG2.

The invention therefore provides a novel approach to regulating β-amyloid production in brain tissue. Without being bound by a specific hypothesis, the inventors believe that inhibition of ABC transporter expression, and/or inhibition of the transport activity of ABC transporters, will be associated with a decrease in APP expression. Because APP is the immediate precursor of β-amyloid, the component of amyloid plaques in the brains of patients with Alzheimer's disease, decreasing APP expression will result in the reduction in the levels of β-amyloid, leading to a prevention or amelioration of amyloid plaque build-up.

Although one goal of the invention is the inhibition of APP expression in humans, the present results also find utility by providing an experimental model for Alzheimer's disease. Brain cells of an animal, preferably a mammal, are transfected with a construct encoding an ABC transporter. The cells will exhibit an increased level of APP expression. The animal model

will be useful for testing methods and agents as candidates for modulating or altering the ABC transporter-related expression of APP.

The invention is not limited to any particular method of altering the expression of an ABC transporter such as ABCB9, ABCG4, and ABCG1, and can include regulation of the promoter region of the ABC transporter gene, and use of antisense oligonucleotides or ribozymes capable of specifically binding to polynucleotides encoding the ABC transporter.

2. Polypeptide Compositions

As used herein, the term "polypeptide" " is used in its conventional meaning, *i.e.*, as a sequence of amino acids. The polypeptides are not limited to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide, and such terms may be used interchangeably herein unless specifically indicated otherwise. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. A polypeptide may be an entire protein, or a subsequence thereof. Particular polypeptides of interest in the context of this invention are amino acid subsequences comprising epitopes, *i.*e., antigenic determinants substantially responsible for the immunogenic properties of a polypeptide and being capable of evoking an immune response.

Particularly illustrative polypeptides of the present invention comprise those encoded by a polynucleotide sequence set forth in any one of SEQ ID NOs: 1-5, or a sequence that hybridizes under moderately stringent conditions, or, alternatively, under highly stringent conditions, to a polynucleotide sequence set forth in any one of SEQ ID NOs: 1-5. Certain other illustrative polypeptides of the invention comprise amino acid sequences as set forth in any one of SEQ ID NOs: 6-10.

In certain preferred embodiments, the polypeptides of the invention are immunogenic, *i.e.*, they react detectably within an immunoassay (such as an ELISA or T-cell stimulation assay) with antisera and/or T-cells from a patient with Alzheimer's disease.

Screening for immunogenic activity can be performed using techniques well known to the skilled artisan. For example, such screens can be performed using methods such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In one illustrative example, a polypeptide may be immobilized on a solid support and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound sera may then be removed and bound antibodies detected using, for example, ^{125}I-labeled Protein A.

As would be recognized by the skilled artisan, immunogenic portions of the polypeptides disclosed herein are also encompassed by the present invention. An "immunogenic portion," as used herein, is a fragment of an immunogenic polypeptide of the invention that itself is immunologically reactive (*i.e.,* specifically binds) with the B-cells and/or T-cell surface antigen receptors that recognize the polypeptide. Immunogenic portions may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or clones. As used herein, antisera and antibodies are "antigen-specific" if they specifically bind to an antigen (*i.e.,* they react with the protein in an ELISA or other immunoassay, and do not react detectably with unrelated proteins). Such antisera and antibodies may be prepared as described herein, and using well-known techniques.

In one preferred embodiment, an immunogenic portion of a polypeptide of the present invention is a portion that reacts with antisera and/or T-cells at a level that is not substantially less than the reactivity of the full-length polypeptide (*e.g.,* in an ELISA and/or T-cell reactivity assay). Preferably, the level of immunogenic activity of the immunogenic portion is at least about 50%, preferably at least about 70% and most preferably greater than about 90% of the immunogenicity for the full-length polypeptide. In some instances, preferred immunogenic portions will be identified that have a level of immunogenic activity greater than that of the corresponding full-length polypeptide, *e.g.*, having greater than about 100% or 150% or more immunogenic activity.

In certain other embodiments, illustrative immunogenic portions may include peptides in which an N-terminal leader sequence and/or transmembrane domain have been deleted. Other illustrative immunogenic portions will contain a small N- and/or C-terminal deletion (*e.g.,* 1-30 amino acids, preferably 5-15 amino acids), relative to the mature protein.

In another embodiment, a polypeptide composition of the invention may also comprise one or more polypeptides that are immunologically reactive with T cells and/or antibodies generated against a polypeptide of the invention, particularly a polypeptide having an amino acid sequence disclosed herein, or to an immunogenic fragment or variant thereof.

In another embodiment of the invention, polypeptides are provided that comprise one or more polypeptides that are capable of eliciting T cells and/or antibodies that are immunologically reactive with one or more polypeptides described herein, or one or more polypeptides encoded by contiguous nucleic acid sequences contained in the polynucleotide sequences disclosed herein, or immunogenic fragments or variants thereof, or to one or more nucleic acid sequences which hybridize to one or more of these sequences under conditions of moderate to high stringency.

The present invention, in another aspect, provides polypeptide fragments comprising at least about 5, 10, 15, 20, 25, 50, or 100 contiguous amino acids, or more, including all intermediate lengths, of a polypeptide compositions set forth herein, such as those set forth in SEQ ID NOs: 6-10, or those encoded by a polynucleotide sequence set forth in a sequence of SEQ ID NOs: 1-5.

In another aspect, the present invention provides variants of the polypeptide compositions described herein. Polypeptide variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity (determined as described below), along its length, to a polypeptide sequences set forth herein.

In one preferred embodiment, the polypeptide fragments and variants provided by the present invention are immunologically reactive with an antibody and/or T-cell that reacts with a full-length polypeptide specifically set forth herein.

In another preferred embodiment, the polypeptide fragments and variants provided by the present invention exhibit a level of immunogenic activity of at least about 50%, preferably at least about 70%, and most preferably at least about 90% or more of that exhibited by a full-length polypeptide sequence specifically set forth herein.

In another preferred embodiment, the polypeptide fragments and variants provided by the present invention are involved in the modulation (*i.e.,* either increase or decrease) of APP expression.

A polypeptide "variant," as the term is used herein, is a polypeptide that typically differs from a polypeptide specifically disclosed herein in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences of the invention and evaluating their immunogenic activity as described herein and/or using any of a number of techniques well known in the art.

For example, certain illustrative variants of the polypeptides of the invention include those in which one or more portions, such as an N-terminal leader sequence or transmembrane domain, have been removed. Other illustrative variants include variants in which a small portion (*e.g.,* 1-30 amino acids, preferably 5-15 amino acids) has been removed from the N- and/or C-terminal of the mature protein.

In many instances, a variant will contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. As described above, modifications may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics, *e.g.*, with immunogenic characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, immunogenic variant or portion of a polypeptide of the invention, one skilled in the art will typically change one or more of the codons of the encoding DNA sequence according to Table 1.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.

TABLE 1

Amino Acids			Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic acid	Asp	D	GAC	GAU				
Glutamic acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					
Tyrosine	Tyr	Y	UAC	UAU				

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of its

hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (–0.4); threonine (–0.7); serine (–0.8); tryptophan (–0.9); tyrosine (–1.3); proline (–1.6); histidine (–3.2); glutamate (–3.5); glutamine (–3.5); aspartate (–3.5); asparagine (–3.5); lysine (–3.9); and arginine (–4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101 (specifically incorporated herein by reference in its entirety), states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (–0.4); proline (–0.5 ± 1); alanine (–0.5); histidine (–0.5); cysteine (–1.0); methionine (–1.3); valine (–1.5); leucine (–1.8); isoleucine (–1.8); tyrosine (–2.3); phenylalanine (–2.5); tryptophan (–3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and

include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

In addition, any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine and uridine.

Amino acid substitutions may further be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or alternatively, contain nonconservative changes. In a preferred embodiment, variant polypeptides differ from a native sequence by substitution, deletion or addition of five amino acids or fewer. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydropathic nature of the polypeptide.

As noted above, polypeptides may comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (*e.g.,* poly-His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

When comparing polypeptide sequences, two sequences are said to be "identical" if the sequence of amino acids in the two sequences is the same when aligned for maximum

correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenes pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS 5*:151-153; Myers, E.W. and Muller W. (1988) *CABIOS 4*:11-17; Robinson, E.D. (1971) *Comb. Theor 11*:105; Saitou, N. Nei, M. (1987) *Mol. Biol. Evol. 4*:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad., Sci. USA 80*:726-730.

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., *Nucl. Acids Res. 25*:3389-3402 (1977) and Altschul et al. *J. Mol.*

Biol. 215:403-410 (1990), respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides and polypeptides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. For amino acid sequences, a scoring matrix can be used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment.

In one preferred approach, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

Within other illustrative embodiments, a polypeptide may be a xenogeneic polypeptide that comprises a polypeptide having substantial sequence identity, as described above, to the human polypeptide (also termed autologous antigen) which served as a reference polypeptide, but which xenogeneic polypeptide is derived from a different, non-human species. One skilled in the art will recognize that "self" antigens are often poor stimulators of CD8+ and CD4+ T-lymphocyte responses, and therefore efficient immunotherapeutic strategies directed against tumor polypeptides require the development of methods to overcome immune tolerance to particular self tumor polypeptides. For example, humans immunized with prostase protein from a xenogeneic (non human) origin are capable of mounting an immune response against the

counterpart human protein, *e.g.* the human prostase tumor protein present on human tumor cells. Accordingly, the present invention provides methods for purifying the xenogeneic form of the ABC transporter proteins set forth herein, such as the polypeptides set forth in SEQ ID NOs: 6-10 or those encoded by polynucleotide sequences set forth in SEQ ID NOs: 1-5.

Therefore, one aspect of the present invention provides xenogeneic variants of the polypeptide compositions described herein. Such xenogeneic variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity along their lengths, to a polypeptide sequences set forth herein.

Within other illustrative embodiments, a polypeptide may be a fusion polypeptide that comprises multiple polypeptides as described herein, or that comprises at least one polypeptide as described herein and an unrelated sequence, such as a known ABC transporter protein. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the polypeptide or to enable the polypeptide to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the polypeptide.

Fusion polypeptides may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion polypeptide is expressed as a recombinant polypeptide, allowing the production of increased levels, relative to a non-fused polypeptide, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion polypeptide that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene 40*:39-46 (1985); Murphy et al., *Proc. Natl. Acad. Sci. USA 83*:8258-8262 (1986); U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly, stop codons required to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

The fusion polypeptide can comprise a polypeptide as described herein together with an unrelated immunogenic protein, such as an immunogenic protein capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see*, for example, Stoute et al., *New Engl. J. Med. 336*:86-91 (1997)).

In one preferred embodiment, the immunological fusion partner is derived from a Mycobacterium sp., such as a Mycobacterium tuberculosis-derived Ra12 fragment. Ra12 compositions and methods for their use in enhancing the expression and/or immunogenicity of heterologous polynucleotide/polypeptide sequences is described in U.S. Patent Application

60/158,585, the disclosure of which is incorporated herein by reference in its entirety. Briefly, Ral2 refers to a polynucleotide region that is a subsequence of a *Mycobacterium tuberculosis* MTB32A nucleic acid. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The nucleotide sequence and amino acid sequence of MTB32A have been described (for example, U.S. Patent Application 60/158,585; *see also*, Skeiky et al., *Infection and Immun. 67*:3998-4007 (1999), incorporated herein by reference). C-terminal fragments of the MTB32A coding sequence express at high levels and remain as a soluble polypeptides throughout the purification process. Moreover, Ral2 may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. One preferred Ral2 fusion polypeptide comprises a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. Other preferred Ral2 polynucleotides generally comprise at least about 15 consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, or at least about 300 nucleotides that encode a portion of a Ral2 polypeptide. Ral2 polynucleotides may comprise a native sequence (*i.e.,* an endogenous sequence that encodes a Ral2 polypeptide or a portion thereof) or may comprise a variant of such a sequence. Ral2 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ral2 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ral2 polypeptide or a portion thereof.

Within other preferred embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium Haemophilus influenza B (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.,* the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression

enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemaglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene 43*:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (*see Biotechnology 10*:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion polypeptide. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

Yet another illustrative embodiment involves fusion polypeptides, and the polynucleotides encoding them, wherein the fusion partner comprises a targeting signal capable of directing a polypeptide to the endosomal/lysosomal compartment, as described in U.S. Patent No. 5,633,234. An immunogenic polypeptide of the invention, when fused with this targeting signal, will associate more efficiently with MHC class II molecules and thereby provide enhanced in vivo stimulation of $CD4^+$ T-cells specific for the polypeptide.

Polypeptides of the invention are prepared using any of a variety of well known synthetic and/or recombinant techniques, the latter of which are further described below. Polypeptides, portions and other variants generally less than about 150 amino acids can be generated by synthetic means, using techniques well known to those of ordinary skill in the art. In one illustrative example, such polypeptides are synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem.*

Soc. *85*:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptide compositions (including fusion polypeptides) of the invention are isolated. An "isolated" polypeptide is one that is removed from its original environment. For example, a naturally-occurring protein or polypeptide is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably, such polypeptides are also purified, *e.g.*, are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

Polynucleotide Compositions

The present invention, in other aspects, provides polynucleotide compositions. The terms "DNA" and "polynucleotide" are used essentially interchangeably herein to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. "Isolated," as used herein, means that a polynucleotide is substantially away from other coding sequences, and that the DNA molecule does not contain large portions of unrelated coding DNA, such as large chromosomal fragments or other functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

As will be understood by those skilled in the art, the polynucleotide compositions of this invention can include genomic sequences, extra-genomic and plasmid-encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the hand of man.

As will be also recognized by the skilled artisan, polynucleotides of the invention may be single-stranded (coding or antisense) or double-stranded, and may be DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but

need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or support materials.

Polynucleotides may comprise a native sequence (*i.e.,* an endogenous sequence that encodes a polypeptide/protein of the invention or a portion thereof) or may comprise a sequence that encodes a variant or derivative, preferably and immunogenic variant or derivative, of such a sequence.

Therefore, according to another aspect of the present invention, polynucleotide compositions are provided that comprise some or all of a polynucleotide sequence set forth in any one of SEQ ID NOs: 1-5 complements of a polynucleotide sequence set forth in any one of SEQ ID NOs: 1-5, and degenerate variants of a polynucleotide sequence set forth in any one of SEQ ID NOs: 1-5. In certain preferred embodiments, the polynucleotide sequences set forth herein encode immunogenic polypeptides, as described above.

In other related embodiments, the present invention provides polynucleotide variants having substantial identity to the sequences disclosed herein in SEQ ID NOs: 1-5, for example those comprising at least 70% sequence identity, preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to a polynucleotide sequence of this invention using the methods described herein, (*e.g.*, BLAST analysis using standard parameters, as described below). One skilled in this art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

Typically, polynucleotide variants will contain one or more substitutions, additions, deletions and/or insertions, preferably such that the immunogenicity of the polypeptide encoded by the variant polynucleotide is not substantially diminished relative to a polypeptide encoded by a polynucleotide sequence specifically set forth herein). The term "variants" should also be understood to encompasses homologous genes of xenogenic origin.

In additional embodiments, the present invention provides polynucleotide fragments comprising or consisting of various lengths of contiguous stretches of sequence identical to or complementary to one or more of the sequences disclosed herein. For example,

polynucleotides are provided by this invention that comprise or consist of at least about 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 300, 400, 500 or 1000 or more contiguous nucleotides of one or more of the sequences disclosed herein as well as all intermediate lengths there between. It will be readily understood that "intermediate lengths", in this context, means any length between the quoted values, such as 16, 17, 18, 19, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.; including all integers through 200-500; 500-1,000, and the like. A polynucleotide sequence as described here may be extended at one or both ends by additional nucleotides not found in the native sequence. This additional sequence may consist of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides at either end of the disclosed sequence or at both ends of the disclosed sequence.

In another embodiment of the invention, polynucleotide compositions are provided that are capable of hybridizing under moderate to high stringency conditions to a polynucleotide sequence provided herein, or a fragment thereof, or a complementary sequence thereof. Hybridization techniques are well known in the art of molecular biology. For purposes of illustration, suitable moderately stringent conditions for testing the hybridization of a polynucleotide of this invention with other polynucleotides include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-60°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. One skilled in the art will understand that the stringency of hybridization can be readily manipulated, such as by altering the salt content of the hybridization solution and/or the temperature at which the hybridization is performed. For example, in another embodiment, suitable highly stringent hybridization conditions include those described above, with the exception that the temperature of hybridization is increased, *e.g.*, to 60-65°C or 65-70°C.

In certain preferred embodiments, the polynucleotides described above, *e.g.*, polynucleotide variants, fragments and hybridizing sequences, encode polypeptides that are immunologically cross-reactive with a polypeptide sequence specifically set forth herein. In other preferred embodiments, such polynucleotides encode polypeptides that have a level of immunogenic activity of at least about 50%, preferably at least about 70%, and more preferably at least about 90% of that for a polypeptide sequence specifically set forth herein.

The polynucleotides of the present invention, or fragments thereof, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, illustrative polynucleotide segments with total lengths of about 10,000, about 5000, about 3000, about 2,000, about 1,000, about 500, about 200, about 100, about 50 base pairs in length, and the like, (including all intermediate lengths) are contemplated to be useful in many implementations of this invention.

When comparing polynucleotide sequences, two sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenes pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS 5*:151-153; Myers, E.W. and Muller W. (1988) *CABIOS 4*:11-17; Robinson, E.D. (1971) *Comb. Theor 11*:105; Santou, N. Nes, M. (1987) *Mol. Biol. Evol. 4*:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical*

Taxonomy, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad., Sci. USA 80*:726-730.

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., *Nucl. Acids Res.* 25:3389-3402 (1977) and Altschul et al. *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. In one illustrative example, cumulative scores can be calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments, (B) of 50, expectation (E) of 10, M=5, N=-4 and a comparison of both strands.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or

deletions (*i.e.,* gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.,* the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, is employed for the preparation of immunogenic variants and/or derivatives of the polypeptides described herein. By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provides a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter,

decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed polynucleotide sequences to alter one or more properties of the encoded polypeptide, such as the immunogenicity of a polypeptide vaccine. The techniques of site-specific mutagenesis are well-known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector that includes within its sequence a DNA sequence that encodes the desired peptide. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful

species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy et al., 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis et al., 1982, each incorporated herein by reference, for that purpose.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (*see*, for example, Watson, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are provided by U. S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety.

In another approach for the production of polynucleotide variants of the present invention, recursive sequence recombination, as described in U.S. Patent No. 5,837,458, may be employed. In this approach, iterative cycles of recombination and screening or selection are performed to "evolve" individual polynucleotide variants of the invention having, for example, enhanced immunogenic activity.

In other embodiments of the present invention, the polynucleotide sequences provided herein can be advantageously used as probes or primers for nucleic acid hybridization. As such, it is contemplated that nucleic acid segments that comprise or consist of a sequence region of at least about a 15 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 15 nucleotide long contiguous sequence disclosed herein will find

particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000 (including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to a sequence of interest will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are also envisioned, such as the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Polynucleotide molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so (including intermediate lengths as well), identical or complementary to a polynucleotide sequence disclosed herein, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. This would allow a gene product, or fragment thereof, to be analyzed, both in diverse cell types and also in various bacterial cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 15 and about 100 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

The use of a hybridization probe of about 15-25 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 15 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 25 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All that is required is to review the sequences set forth herein, or to any continuous portion of the sequences, from about 15-25 nucleotides in length up to and including

the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors. For example, one may wish to employ primers from towards the termini of the total sequence.

Small polynucleotide segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patent 4,683,202 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

The nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of the entire gene or gene fragments of interest. Depending on the application envisioned, one will typically desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by a salt concentration of from about 0.02 M to about 0.15 M salt at temperatures of from about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating related sequences.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent (reduced stringency) hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ salt conditions such as those of from about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased

temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

According to another embodiment of the present invention, polynucleotide compositions comprising antisense oligonucleotides are provided. Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, provide a therapeutic approach by which a disease can be treated by inhibiting the synthesis of proteins that contribute to the disease. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalactauronase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal $GABA_A$ receptor and human EGF (Jaskulski et al., *Science 240*(4858):1544-6 (1988); Vasanthakumar and Ahmed, *Cancer Commun.*, *1*(4):225-32 (1989); Peris et al., *Brain Res Mol* 57(2):310-20 (1998); U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Antisense constructs have also been described that inhibit and can be used to treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

Therefore, in certain embodiments, the present invention provides oligonucleotide sequences that comprise all, or a portion of, any sequence that is capable of specifically binding to polynucleotide sequence described herein, or a complement thereof. In one embodiment, the antisense oligonucleotides comprise DNA or derivatives thereof. In another embodiment, the oligonucleotides comprise RNA or derivatives thereof. In a third embodiment, the oligonucleotides are modified DNAs comprising a phosphorothioated modified backbone. In a fourth embodiment, the oligonucleotide sequences comprise peptide nucleic acids or derivatives thereof. In each case, preferred compositions comprise a sequence region that is complementary, and more preferably substantially-complementary, and even more preferably, completely complementary to one or more portions of polynucleotides disclosed herein. Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen

target sequence and determination of secondary structure, T_m, binding energy, and relative stability. Antisense compositions may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those which are at or near the AUG translation initiation codon, and those sequences which are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software and/or the BLASTN 2.0.5 algorithm software (Altschul et al., *Nucleic Acids Res.* *25*(17):3389-402 (1997)).

The use of an antisense delivery method employing a short peptide vector, termed MPG (27 residues), is also contemplated. The MPG peptide contains a hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain from the nuclear localization sequence of SV40 T-antigen (Morris et al., *Nucleic Acids Res.* *25*(14):2730-6 (1997)). It has been demonstrated that several molecules of the MPG peptide coat the antisense oligonucleotides and can be delivered into cultured mammalian cells in less than 1 hour with relatively high efficiency (90%). Further, the interaction with MPG strongly increases both the stability of the oligonucleotide to nuclease and the ability to cross the plasma membrane.

According to another embodiment of the invention, the polynucleotide compositions described herein are used in the design and preparation of ribozyme molecules for inhibiting expression of the tumor polypeptides and proteins of the present invention in tumor cells. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci U S A. 1987 Dec; 84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). For example, a large number of ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech et al., *Cell* *27*(3 Pt 2):487-96 (1981); Michel and Westhof, *J Mol Biol.*, *216*(3):585-610 (1990); Reinhold-Hurek and Shub, *Nature*, *357*(6374):173-6 (1992)). This specificity has been attributed to the requirement that the substrate bind via specific base-

pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Six basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf et al., *Proc Natl Acad Sci U S A 89*(16):7305-9 (1992)). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis δ virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi et al. *Nucleic Acids Res. 20*(17):4559-65 (1992). Examples of hairpin motifs are described by Hampel

et al., Eur. Pat. Appl. Publ. No. EP 0360257, Hampel and Tritz, *Biochemistry 28*(12):4929-33 (1989); Hampel et al., *Nucleic Acids Res.* 18(2):299-304 (1990) and U. S. Patent 5,631,359. An example of the hepatitis δ virus motif is described by Perrotta and Been, *Biochemistry 31*(47):11843-52 (1992); an example of the RNaseP motif is described by Guerrier-Takada et al., *Cell* 35(3 Pt 2):849-57 (1983); Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, *Cell 61*(4):685-96(1990); Saville and Collins, *Proc Natl Acad Sci U S A, 88*(19):8826-30 (1991); Collins and Olive, *Biochemistry 32*(11):2795-9 (1993)); and an example of the Group I intron is described in (U. S. Patent 4,987,071). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U. S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

Sullivan et al. (Int. Pat. Appl. Publ. No. WO 94/02595) describes the general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to,

encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Another means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells. Ribozymes expressed from such promoters have been shown to function in mammalian cells. Such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

In another embodiment of the invention, peptide nucleic acids (PNAs) compositions are provided. PNA is a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone (Good and Nielsen, Antisense Nucleic Acid Drug Dev. 1997 7(4) 431-37). PNA can be utilized in a number of methods that traditionally have used RNA or DNA. Often PNA sequences perform better in techniques than the corresponding RNA or DNA sequences and have utilities that are not inherent to RNA or DNA. A review of PNA including

methods of making, characteristics of, and methods of using, is provided by Corey (*Trends Biotechnol 15*(6):224-9 (1997)). As such, in certain embodiments, one may prepare PNA sequences that are complementary to one or more portions of the ACE mRNA sequence, and such PNA compositions may be used to regulate, alter, decrease, or reduce the translation of ACE-specific mRNA, and thereby alter the level of ACE activity in a host cell to which such PNA compositions have been administered.

PNAs have 2-aminoethyl-glycine linkages replacing the normal phosphodiester backbone of DNA (Nielsen et al., *Science 254*(5037):1497-500 (1991); Hanvey et al., *Science 258*(5087):1481-5 (1992); Hyrup and Nielsen, *Bioorg Med Chem. 4*(1):5-23 (1996)). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules; secondly, PNAs are achiral, which avoids the need to develop a stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc or Fmoc protocols for solid-phase peptide synthesis, although other methods, including a modified Merrifield method, have been used.

PNA monomers or ready-made oligomers are commercially available from PerSeptive Biosystems (Framingham, MA). PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols (Norton et al., *Bioorg Med Chem. 3*(4):437-45 (1995)). The manual protocol lends itself to the production of chemically modified PNAs or the simultaneous synthesis of families of closely related PNAs.

As with peptide synthesis, the success of a particular PNA synthesis will depend on the properties of the chosen sequence. For example, while in theory PNAs can incorporate any combination of nucleotide bases, the presence of adjacent purines can lead to deletions of one or more residues in the product. In expectation of this difficulty, it is suggested that, in producing PNAs with adjacent purines, one should repeat the coupling of residues likely to be added inefficiently. This should be followed by the purification of PNAs by reverse-phase high-pressure liquid chromatography, providing yields and purity of product similar to those observed during the synthesis of peptides.

Modifications of PNAs for a given application may be accomplished by coupling amino acids during solid-phase synthesis or by attaching compounds that contain a carboxylic

acid group to the exposed N-terminal amine. Alternatively, PNAs can be modified after synthesis by coupling to an introduced lysine or cysteine. The ease with which PNAs can be modified facilitates optimization for better solubility or for specific functional requirements. Once synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry. Several studies have made and utilized modifications of PNAs (for example, Norton et al. *Bioorg Med Chem. 3*(4):437-45 (1995); Petersen et al. *J Pept Sci. 1*(3):175-83 (1995); Orum et al., *Biotechniques 19*(3):472-80 (1995); Footer et al. *Biochemistry 35*(33):10673-9 (1996); Griffith et al. *Nucleic Acids Res.* 23(15):3003-8 (1995); Pardridge et al., *Proc Natl Acad Sci U S A 92*(12):5592-6 (1995); Boffa et al. *Proc Natl Acad Sci U S A 92*(6):1901-5 (1995); Gambacorti-Passerini et al., *Blood 88*(4):1411-7 (1996); Armitage et al. *Proc Natl Acad Sci U S A, 94*(23):12320-5 (1997); Seeger et al. *Biotechniques, 23*(3):512-7 (1997)). U.S. Patent No. 5,700,922 discusses PNA-DNA-PNA chimeric molecules and their uses in diagnostics, modulating protein in organisms, and treatment of conditions susceptible to therapeutics.

Methods of characterizing the antisense binding properties of PNAs are discussed in Rose (*Anal Chem. 65*(24):3545-9 (1993)) and Jensen et al. (*Biochemistry, 36*(16):5072-7 (1997)). Rose uses capillary gel electrophoresis to determine binding of PNAs to their complementary oligonucleotide, measuring the relative binding kinetics and stoichiometry. Similar types of measurements were made by Jensen et al. using BIAcore™ technology.

Other applications of PNAs that have been described and will be apparent to the skilled artisan include use in DNA strand invasion, antisense inhibition, mutational analysis, enhancers of transcription, nucleic acid purification, isolation of transcriptionally active genes, blocking of transcription factor binding, genome cleavage, biosensors, *in situ* hybridization, and the like.

4. Antisense Oligonucleotides

One aspect of the invention therefore pertains to isolated nucleic acid molecules that are antisense to ABC transporter polynucleotides. An "antisense" nucleic acid comprises a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can hydrogen bond to a sense

nucleic acid. The antisense nucleic acid can be complementary to an entire ABC transporter coding strand, or to only a portion thereof. In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding an ABC transporter. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding an ABC transporter. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

The polynucleotide sequences of ABC transporters ABCB9, ABCB1, ABCA2 ABCG4, and ABCG1 are shown in SEQ ID NO:1, 2, 3 4 and 5, respectively. Given the coding strand sequences encoding ABC transporter, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of ABC transporter mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of ABC transporter mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of ABC transporter mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides or more in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, β-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-

methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5- oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding an ABC transporter protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementary to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule which binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention include direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient intracellular concentrations of the antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific

double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier et al., *Nucleic Acids. Res. 15*:6625-6641 (1987)). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue et al., *Nucleic Acids Res. 15*:6131-6148 (1987)) or a chimeric RNA-DNA analogue (Inoue et al., *FEBS Lett. 215*:327-330 (1987)).

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity which are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach, *Nature 334*:585-591 (1988))) can be used to catalytically cleave ABC transporter mRNA transcripts to thereby inhibit translation of ABC transporter mRNA. A ribozyme having specificity for an ABC-encoding nucleic acid can be designed based upon the nucleotide sequence of an ABC transporter cDNA disclosed herein (*i.e.*, SEQ ID NO:1). For example, a derivative of a *Tetrahymena* L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in an ABC-encoding mRNA. *See, e.g.*, Cech et al. U.S. Patent No. 4,987,071; and Cech et al. U.S. Patent No. 5,116,742. Alternatively, ABC transporter mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. *See, e.g.*, Bartel, D. and Szostak, J.W., *Science 261*:1411-1418 (1993).

5. Regulatory Regions of ABC Transporter Gene

ABC transporter gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the ABC transporter (*e.g.*, the ABC transporter promoter and/or enhancers) to form triple helical structures that prevent transcription of the ABC transporter gene in target cells. *See generally*, Helene, C., *Anticancer Drug Des. 6(6)*:569-84 (1991); Helene, C. et al., *Ann. N.Y. Acad. Sci. 660*:27-36 (1992); and Maher, L.J., *Bioassays 14(12)*:807-15 (1992).

6. Expression Constructs, Host Cells and Transgenic Animals

The invention further provides a recombinant expression vector comprising a DNA molecule encoding an ABC transporter cloned into the expression vector in an antisense

orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to ABC transporter mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types. For instance, viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which an ABC transporter nucleic acid molecule of the invention is introduced, *e.g.*, an ABC transporter nucleic acid molecule within a recombinant expression vector or an ABC transporter nucleic acid molecule containing sequences which allow it to homologously recombine into a specific site of the host cell's genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, an ABC transporter protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms

"transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding an ABC transporter protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

The host cells of the invention can also be used to produce non-human transgenic animals. For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which ABC-coding sequences have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous ABC transporter sequences have been introduced into their genome or homologous recombinant animals in which endogenous ABC transporter sequences have been altered. Such animals are useful for studying the function and/or activity of an ABC transporter and for identifying and/or evaluating modulators of ABC transporter activity. According to the invention, the animals are useful for regulating the expression of APP by modulating ABC transporter expression. As used herein, a "transgenic animal" is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human primates, sheep, dogs, cows, goats, chickens,

amphibians, and the like. A transgene is exogenous DNA which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. As used herein, a "homologous recombinant animal" is a non-human animal, preferably a mammal, more preferably a mouse, in which an endogenous ABC transporter gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, *e.g.*, an embryonic cell of the animal, prior to development of the animal.

A transgenic animal of the invention can be created by introducing an ABC-encoding nucleic acid into the male pronuclei of a fertilized oocyte, *e.g.*, by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. An ABC transporter cDNA sequence can be introduced as a transgene into the genome of a non-human animal. Alternatively, a nonhuman homologue of a human ABC transporter gene, such as a mouse or rat ABC transporter gene, can be used as a transgene. Alternatively, an ABC transporter gene homologue, such as another ABC transporter family member, can be isolated based on hybridization to ABC transporter cDNA sequence and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be operably linked to an ABC transporter transgene to direct expression of an ABC transporter protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Patent No. 4,873,191 by Wagner et al. and in Hogan, B., *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of an ABC transporter transgene in its genome and/or expression of ABC transporter mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding an ABC transporter protein can further be bred to other transgenic

animals carrying other transgenes, for example, animals carrying a transgene encoding a neurotoxic polypeptide such as β-amyloid.

To create a homologous recombinant animal, a vector is prepared which contains at least a portion of an ABC transporter gene into which a deletion, addition or substitution has been introduced to thereby alter, *e.g.*, functionally disrupt, the ABC transporter gene. The ABC transporter gene can be a human gene, but more preferably, is a non-human homologue of a human ABC transporter gene (*e.g.*, a cDNA isolated by stringent hybridization with the nucleotide sequence of a human ABC transporter polynucleotide). For example, a mouse ABC transporter gene can be used to construct a homologous recombination nucleic acid molecule, *e.g.*, a vector, suitable for altering an endogenous ABC transporter gene in the mouse genome. In a preferred embodiment, the homologous recombination nucleic acid molecule is designed such that, upon homologous recombination, the endogenous ABC transporter gene is functionally disrupted (*i.e.*, no longer encodes a functional protein; also referred to as a "knock out" vector). Alternatively, the homologous recombination nucleic acid molecule can be designed such that, upon homologous recombination, the endogenous ABC transporter gene is mutated or otherwise altered but still encodes functional protein (*e.g.*, the upstream regulatory region can be altered to thereby alter the expression of the endogenous ABC transporter protein). In the homologous recombination nucleic acid molecule, the altered portion of the ABC transporter gene is flanked at its 5' and 3' ends by additional nucleic acid sequence of the ABC transporter gene to allow for homologous recombination to occur between the exogenous ABC transporter gene carried by the homologous recombination nucleic acid molecule and an endogenous ABC transporter gene in a cell, *e.g.*, an embryonic stem cell. The additional flanking ABC transporter nucleic acid sequence is of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the homologous recombination nucleic acid molecule (*see*, *e.g.*, Thomas, K.R. and Capecchi, M. R., *Cell 51*:503 (1987) for a description of homologous recombination vectors). The homologous recombination nucleic acid molecule is introduced into a cell, *e.g.*, an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced ABC transporter gene has homologously recombined with the endogenous ABC

transporter gene are selected (*see e.g.*, Li, E. et al., *Cell 69*:915 (1992)). The selected cells can then injected into a blastocyst of an animal (*e.g.*, a mouse) to form aggregation chimeras (*see e.g.*, Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination nucleic acid molecules, *e.g.*, vectors, or homologous recombinant animals are described further in Bradley, A., *Current Opinion in Biotechnology 2*:823-829 (1991) and in PCT International Publication Nos.: WO 90/11354 by Le Mouellec et al.; WO 91/01140 by Smithies et al.; WO 92/0968 by Zijlstra et al.; and WO 93/04169 by Berns et al.

In another embodiment, transgenic non-human animals can be produced which contain selected systems which allow for regulated expression of the transgene. One example of such a system is the *cre/loxP* recombinase system of bacteriophage P1. For a description of the *cre/loxP* recombinase system, *see*, *e.g.*, Lakso et al., *Proc. Natl. Acad. Sci. USA 89*:6232-6236 (1992). Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al., *Science 251*:1351-1355 (1991). If a *cre/loxP* recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the *Cre* recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, *e.g.*, by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut, I. et al., *Nature 385*:810-813 (1997) and PCT International Publication Nos. WO 97/07668 and WO 97/07669. In brief, a cell, *e.g.*, a somatic cell, from the transgenic animal can be isolated and induced to exit the growth cycle and enter G_0 phase. The quiescent cell can then be fused, *e.g.*, through the use of electrical pulses, to an enucleated oocyte from an animal of the same species from which the quiescent cell is

isolated. The reconstructed oocyte is then cultured such that it develops to morula or blastocyte and then transferred to pseudopregnant female foster animal. The offspring borne of this female foster animal will be a clone of the animal from which the cell, *e.g.*, the somatic cell, is isolated.

7. Pharmaceutical Compositions

The ABC transporter nucleic acid molecules, fragments of ABC transporter proteins, anti-ABC transporter antibodies (also referred to herein as "active compounds"), expressible nucleic acids encoding ABC transporters (or fragments thereof), or any compound identified as a modulator of an ABC transporter (as described herein) can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or

bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a fragment of an ABC transporter protein or an anti-ABC transporter antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic ABC transporter dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable,

biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.,* for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The

dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (*see* U.S. Patent 5,328,470) or by stereotactic injection (*see e.g.*, Chen et al., *Proc. Natl. Acad. Sci. USA 91*:3054-3057 (1994)). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

8. Uses and Methods of the Invention

The biological materials and methods described herein can be used in one or more of the following methods: a) screening assays; b) predictive medicine (*e.g.*, diagnostic assays, prognostic assays, monitoring clinical trials, and pharmacogenetics); and c) methods of treatment (*e.g.*, therapeutic and prophylactic).

Isolated nucleic acid molecules can be used, for example, to express ABC transporter protein (*e.g.*, via a recombinant expression vector in a host cell in gene therapy applications), to detect ABC transporter mRNA (*e.g.*, in a biological sample) or a genetic alteration in an ABC transporter gene, and to modulate ABC transporter activity, as described

further below. The ABC transporter proteins can be used to treat disorders characterized by insufficient or excessive production of an ABC transporter substrate such as β-amyloid, or production of ABC transporter inhibitors. In addition, the ABC transporter proteins can be used to screen for naturally occurring ABC transporter substrates, to screen for drugs or compounds which modulate ABC transporter activity, as well as to treat disorders characterized by insufficient or excessive production of ABC transporter protein or production of ABC transporter protein forms which have decreased, aberrant or unwanted activity compared to ABC transporter wild type protein. Moreover, the anti-ABC transporter antibodies of the invention can be used to detect and isolate ABC transporter proteins, regulate the bioavailability of ABC transporter proteins, and modulate ABC transporter activity.

The current invention also identifies compounds that can be used in both *in vitro* screening assays and cell based assays. *In vitro* screening assays include vesicular assays, assays using purified protein, assays using reconstituted protein, and the like. Some of these compounds are able to modulate the expression and/or activity of ABC transporters via either directly binding to the ABC transporter or via effects of the compounds on substances, which in turn affect the expression, and/or activity of the ABC transporter. Other compounds may exert their effects by binding directly to an ABC transporter of the current invention, resulting in either: 1) direct modulation of the activity of the ABC transporter, 2) alterations in binding and/or transport of the substrate which binds to a ABC transporter, or 3) alterations in the interaction of the ABC transporter with other proteins. Examples of such molecules include, but are not limited to, allosteric modulators, small molecules, and peptides.

A. Screening Assays

The invention provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) which bind to ABC transporter proteins, have a stimulatory or inhibitory effect on, for example, ABC transporter expression or ABC transporter activity, or have a stimulatory or inhibitory effect on, for example, the expression or

activity of an ABC transporter substrate. In specific embodiments, the end-point of the assay is the measurement of APP expression.

In one embodiment, the invention provides assays for screening candidate or test compounds which are modulators or substrates of an ABC transporter protein or polypeptide or biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test compounds which bind to or modulate the activity of an ABC transporter protein or polypeptide or biologically active portion thereof. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including but not limited to: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S., *Anticancer Drug Des. 12*:145 (1997)).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al., *Proc. Natl. Acad. Sci. U.S.A. 90*:6909 (1993); Erb et al., *Proc. Natl. Acad. Sci. USA 91*:11422 (1994); Zuckermann et al., *J. Med. Chem. 37*:2678 (1994); Cho et al., *Science 261*:1303 (1993); Carrell et al., *Angew. Chem. Int. Ed. Engl. 33*:2059 (1994); Carrell et al., *Angew. Chem. Int. Ed. Engl. 33*:2061 (1994); and in Gallop et al., *J. Med. Chem. 37*:1233 (1994).

Libraries of compounds may be presented in solution (*e.g.*, Houghten, *Biotechniques 13*:412-421 (1992)), or on beads (Lam, *Nature 354*:82-84 (1991)), chips (Fodor, *Nature 364*:555-556 (1993)), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull et al., *Proc Natl Acad Sci USA 89*:1865-1869 (1992)) or on phage (Scott and Smith, *Science 249*:386-390 (1990)); (Devlin, *Science 249*:404-406 (1990)); (Cwirla et al., *Proc. Natl. Acad. Sci. 87*:6378-6382 (1990)); (Felici, *J. Mol. Biol. 222*:301-310 (1991)); (Ladner *supra.*).

In one embodiment, an assay is a cell-based assay in which a cell which expresses an ABC transporter protein or biologically active portion thereof is contacted with a test

compound and the ability of the test compound to modulate ABC transporter activity and/or expression, and thereby APP expression, is determined. Determining the ability of the test compound to modulate ABC transporter activity can be accomplished by monitoring, for example, cellular transport of organic anions, organic cations, cytotoxic anti-cancer drugs, intracellular calcium, potassium, phosphatidylcholine, sodium concentration, neuronal membrane depolarization, a neurotoxic polypeptide (*e.g.*, β-amyloid), or the activity of an ABC transporter-regulated transcription factor. The cell, for example, can be of mammalian origin, *e.g.*, a neuronal cell. The ability of the test compound to modulate ABC transporter binding to a substrate or to bind to ABC transporter can also be determined. Determining the ability of the test compound to modulate ABC transporter binding to a substrate can be accomplished, for example, by coupling the ABC transporter substrate with a radioisotope or enzymatic label such that binding of the ABC transporter substrate to ABC transporter can be determined by detecting the labeled ABC transporter substrate in a complex. Determining the ability of the test compound to bind ABC transporter can be accomplished, for example, by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to ABC transporter can be determined by detecting the labeled ABC transporter compound in a complex. For example, compounds (*e.g.*, ABC transporter substrates) can be labeled with ^{125}I, ^{35}S, ^{14}C, ^{33}P, ^{32}P, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, compounds can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

In one embodiment, suitable compounds include, but are not limited to, verapamil, desmethoxyverapamil, chloroquine, quinine, chinchonidine, primaquine, tamoxifen, dihydrocyclosporin, yohimbine, corynanthine, reserpine, physostigmine, acridine, acridine orange, quinacrine, trifluoroperazine chlorpromazine, propanolol, atropine, tryptamine, forskolin, 1,9-dideoxyforskolin, cyclosporin, (US Patent 4,117,118 (1978)), PSC-833 (cyclosporin D, 6-[(2S, 4R, 6E)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid]-(9CI)), [US Patent 5,525,590] [ACS 121584-18-7], Keller et al., *Int J Cancer 50*:593-597 (1992)), RU-486 (17β-hydroxy-11β-[4-dimethylaminophenyl]-17α prop-1-ynyl estra-4, 9-dien-3 one), RU-49953 (17β-hydroxy-11β,

17α-[4-dimethylaminophenyl] - 17α prop-1-ynyl estra-4, 9 dien-3 one), S9778 (6-{4-[2,2-di( )-ethylamino]-1-piperidinyl}-*N,N'*, di-2-propenyl-1,3,5-triazine-2,4-diamine, bismethane sulfonate, [US patent 5,225,411; EP 466586] [ACS # 140945-01-3]; Dhainaut et al., "New triazine derivatives as potent modulators of multidrug resistance," *J Medicinal Chemistry 35*:2481-2496 (1992)), MS-209 (5-[3-[4-(2,2-diphenylacetyl)piperazin-1-yl]-2-hydroxypropoxy]quinoline sesquifumarate, [US patent 5,405,843 (continuation of 5,112,817)], [ACS # 158681-49-3], Sato et al., "Reversal of multidrug resistance by a novel quinoline derivative, MS-209, *Cancer Chemother Pharmacol 35*:271-277 (1995)), MS-073 (Fukazawa et al., European Patent Application 0363212 (1989)), FK-506 (Tanaka et al., M. Physicochemical properties of FK-506, a novel immunosuppressant isolated from *Streptomyces tsukubaensis*" *Transplantation Proceedings. 19(5 Suppl 6)*:11-6, (1987); Naito et al., "Reversal of multidrug resistance by an immunosuppressive agent FK-506," *Cancer Chemother & Pharmacol. 29*:195-200 (1992); Pourtier-Manzanedo et al., "FK-506 (fujimycin) reverses the multidrug resistance of tumor cells in vitro," *Anti-Cancer Drugs 2*:279-83 (1991); Epand & Epand, "The new potent immunosuppressant FK-506 reverses multidrug resistance in Chinese hamster ovary cells," *Anti-Cancer Drug Design 6*:189-93 (1991)), VX-710 (2-peperidinecarboxylic acid, 1-[oxo(3,4,5-trimethoxyphenyl)acetyl]-3-(3-pyridinyl)-1-[3-(3-pyridinyl)propyl]butyl ester [ACS 159997-94-1] [US patent number 5,620,971] Germann et al., "Chemosensitization and drug accumulation effects of VX-710, verapamil, cyclosporin A, MS-209 and GF120918 in multidrug resistance-associated protein MRP" *Anti-Cancer Drugs 8*:41-155 (1997) ; Germann et al., "Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance in vitro" *Anti-Cancer Drugs 8*:125-140 (1997)), VX-853 ([US patent number 5,543,423] [ACS # 190454-58-1), AHC-52 (methyl 2-(N-benzyl-N-methylamino)ethyl-2, 6-dimethyl-4-(2-isopropylpyrazolo[1,5-a]pyridine-3-yl)-1,4-dihyropyridine-3,5-dicarboxylate; [Japanese Patent 63-135381; European Patent 0270926] [ACS 119666-09-0] Shinoda et al., "In vivo circumvention of vincristine resistance in mice with P388 leukemia using a novel compound, AHC-52," *Cancer Res 49*:1722-6 (1989)), GF-120918 (9,10-dihydro-5-methoxy-9-oxo-N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxyisoquinol-2-yl) ethyl]phenyl]-4 acridinecarboxamide,[US patent 5,604,237] [ACS # 143664-11-3] Hyafil et al.,

"In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative," *Cancer Res 53*:4595-4602 (1993)), and XR-9051 (3-[(3Z, 6Z)-6-Benzylidene-1-methyl-2,5-dioxopiperazin-3-ylidenemethyl]-N-[4-[2-(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]phenyl]benzamide hydrochloride, [ACS#57-22-7]).

B. Predictive Medicine

The present invention also pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly, one aspect of the present invention relates to diagnostic assays for determining ABC transporter protein and/or nucleic acid expression as well as ABC transporter activity, such as APP expression, in the context of a biological sample (*e.g.*, cells, tissue) to thereby determine whether an individual is afflicted with a disease or disorder, or is at risk of developing a disorder, associated with aberrant or unwanted ABC transporter expression or activity. The invention also provides for prognostic (or predictive) assays for determining whether an individual is at risk of developing a disorder associated with ABC transporter protein, nucleic acid expression or activity. For example, mutations in an ABC transporter gene can be assayed in a biological sample, such as those described in the Examples. Such assays can be used for prognostic or predictive purpose to thereby prophylactically treat an individual prior to the onset of a disorder characterized by or associated with ABC transporter protein, nucleic acid expression or activity.

Another aspect of the invention pertains to monitoring the influence of agents (*e.g.*, drugs, compounds) on the expression or activity of ABC transporter in clinical trials.

i. Prognostic Assays

The methods described herein can furthermore be utilized to identify subjects having or at risk of developing a disease or disorder associated with aberrant or unwanted ABC transporter expression or activity. As used herein, the term "aberrant" includes an ABC transporter expression or activity which deviates from the wild type ABC transporter expression or activity, resulting in aberrant APP expression. Aberrant expression or activity includes

increased or decreased expression or activity, as well as expression or activity which does not follow the wild type developmental pattern of expression or the subcellular pattern of expression. For example, aberrant ABC transporter expression or activity is intended to include the cases in which a mutation in the ABC transporter gene causes the ABC transporter gene to be under-expressed or over-expressed and situations in which such mutations result in a non-functional ABC transporter protein or a protein which does not function in a wild-type fashion, *e.g.*, a protein which does not interact with an ABC transporter ligand or one which interacts with a non-ABC transporter ligand. In a particular embodiment herein, the ligand is $A\beta_{1-40}$, $A\beta_{1-42}$, or any Aβ released from the cell membrane. As used herein, the term "unwanted" includes an unwanted phenomenon involved in a biological response. For example, the term unwanted includes an ABC transporter expression or activity which is undesirable in a subject.

These assays can be utilized to identify a subject having or at risk of developing a disorder associated with a misregulation in ABC transporter protein activity or nucleic acid expression, which results in aberrant expression of APP in the cell. Alternatively, the prognostic assays can be utilized to identify a subject having or at risk for developing a disorder associated with a misregulation in ABC transporter protein activity or nucleic acid expression. Thus, the present invention provides a method for identifying a disease or disorder associated with aberrant or unwanted ABC transporter expression or activity in which a test sample is obtained from a subject and ABC transporter protein or nucleic acid (*e.g.*, mRNA or genomic DNA) is detected, wherein the presence of ABC transporter protein or nucleic acid is diagnostic for a subject having or at risk of developing a disease or disorder associated with aberrant or unwanted ABC transporter expression or activity. As used herein, a "test sample" refers to a biological sample obtained from a subject of interest. For example, a test sample can be a biological fluid (*e.g.*, serum), cell sample, or tissue.

Furthermore, the prognostic assays described herein can be used to determine whether a subject can be administered an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate) to treat a disease or disorder associated with aberrant or unwanted ABC transporter expression or activity, *e.g.*, a cancer where the cells of the cancer have developed multidrug resistance. Thus, the present

invention provides methods for determining whether a subject can be effectively treated with an agent for a disorder associated with aberrant or unwanted ABC transporter expression or activity in which a test sample is obtained and ABC transporter protein or nucleic acid expression or activity is detected (*e.g.*, wherein the abundance of ABC transporter protein or nucleic acid expression or activity is diagnostic for a subject that can be administered the agent to treat a disorder associated with aberrant or unwanted ABC transporter expression or activity resulting in aberrant APP expression).

The methods of the invention can also be used to detect genetic alterations in an ABC transporter gene, thereby determining if a subject with the altered gene is at risk for a disorder characterized by misregulation in ABC transporter protein activity or nucleic acid expression. In preferred embodiments, the methods include detecting, in a sample of cells from the subject, the presence or absence of a genetic alteration characterized by at least one of an alteration affecting the integrity of a gene encoding an ABC protein, or the mis-expression of the ABC transporter gene. For example, such genetic alterations can be detected by ascertaining the existence of at least one of 1) a deletion of one or more nucleotides from an ABC transporter gene; 2) an addition of one or more nucleotides to an ABC transporter gene; 3) a substitution of one or more nucleotides of an ABC transporter gene, 4) a chromosomal rearrangement of an ABC transporter gene; 5) an alteration in the level of a messenger RNA transcript of an ABC transporter gene, 6) aberrant modification of an ABC transporter gene, such as of the methylation pattern of the genomic DNA, 7) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an ABC transporter gene, 8) a non-wild type level of an ABC-protein, 9) allelic loss of an ABC transporter gene, and 10) inappropriate post-translational modification of an ABC-protein. As described herein, there are a large number of assays known in the art which can be used for detecting alterations in an ABC transporter gene. A preferred biological sample is a tissue or serum sample isolated by conventional means from a subject.

ii. Monitoring of Effects During Clinical Trials

Monitoring the influence of agents (*e.g.*, drugs) on the expression or activity of an ABC transporter protein can be applied not only in ABC transporter drug screening, but also in

clinical trials. For example, the effectiveness of an agent determined by a screening assay as described herein to increase ABC transporter gene expression, protein levels, or upregulate ABC transporter activity, can be monitored in clinical trials of subjects exhibiting decreased ABC transporter gene expression, protein levels, or downregulated ABC transporter activity, such as APP expression in the cell, such as a neuronal cell. Alternatively, the effectiveness of an agent determined by a screening assay to decrease ABC transporter gene expression, or protein levels, or to downregulate ABC transporter activity, can be monitored in clinical trials of subjects exhibiting increased ABC transporter gene expression, protein levels, or upregulated ABC transporter activity, associated with increased APP expression. In such clinical trials, the expression or activity of an ABC transporter gene, and preferably, other genes that have been implicated in, for example, an ABC-associated disorder can be used as a "read out" or markers of the phenotype of a particular cell.

For example, and not by way of limitation, genes, including ABC, that are modulated in cells by treatment with an agent (*e.g.*, compound, drug or small molecule) which modulates ABC transporter activity (*e.g.*, identified in a screening assay as described herein) can be identified. Thus, to study the effect of agents on ABC-associated disorders, for example, in a clinical trial, cells can be isolated and RNA prepared and analyzed for the levels of expression of ABC transporter and other genes implicated in the ABC-associated disorder, respectively. The levels of gene expression (*e.g.*, a gene expression pattern) can be quantified by northern blot analysis or RT-PCR, as described herein, or alternatively by measuring the amount of protein produced, by one of the methods as described herein, or by measuring the levels of activity of ABC transporter or other genes, particularly expression of APP. In this way, the gene expression pattern can serve as a marker, indicative of the physiological response of the cells to the agent. Accordingly, this response state may be determined before, and at various points during treatment of the individual with the agent.

In a preferred embodiment, the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (*e.g.*, an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule, or other drug candidate identified by the screening assays described herein) including the steps of (i) obtaining a pre-administration

sample from a subject prior to administration of the agent; (ii) detecting the level of expression of an ABC transporter protein, mRNA, or genomic DNA in the preadministration sample; (iii) obtaining one or more post-administration samples from the subject; (iv) detecting the level of expression or activity of the ABC transporter protein, mRNA, or genomic DNA in the post-administration samples; (v) comparing the level of expression or activity of the ABC transporter protein, mRNA, or genomic DNA in the pre-administration sample with the ABC transporter protein, mRNA, or genomic DNA in the post administration sample or samples; and (vi) altering the administration of the agent to the subject accordingly. For example, increased administration of the agent may be desirable to increase the expression or activity of ABC transporter to higher levels than detected, *i.e.*, to increase the effectiveness of the agent. Alternatively, decreased administration of the agent may be desirable to decrease expression or activity of ABC transporter to lower levels than detected, *i.e.* to decrease the effectiveness of the agent. According to such an embodiment, ABC transporter expression or activity may be used as an indicator of the effectiveness of an agent, even in the absence of an observable phenotypic response.

C. Methods of Treatment

The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disorder or having a disorder associated with aberrant or unwanted ABC transporter expression or activity, specifically release of Aβ from the cell membrane. With regards to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics", as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers the study of how a patient's genes determine his or her response to a drug (*e.g.*, a patient's "drug response phenotype", or "drug response genotype".) Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the ABC transporter molecules of the present invention or ABC transporter modulators

according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

i. Prophylactic Methods

In one aspect, the invention provides a method for preventing in a subject, a disease or condition associated with an aberrant or unwanted ABC transporter expression or activity, by administering to the subject an ABC transporter or an agent which modulates ABC transporter expression or at least one ABC transporter activity, wherein the treatment leads to a correction of aberrant APP expression. Subjects at risk for a disease which is caused or contributed to by aberrant or unwanted ABC transporter expression or activity can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein, such as elevated cellular APP expression. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the ABC transporter aberrancy, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of ABC transporter aberrancy, for example, an ABC transporter agonist or ABC transporter antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

ii. Therapeutic Methods

Another aspect of the invention pertains to methods of modulating ABC transporter expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the invention involves contacting a cell with an agent that modulates expression of or transport by an ABC transporter protein associated with the cell. An agent that modulates ABC transporter protein activity or expression can be an agent as described herein, such as a nucleic acid or a protein, a naturally-occurring target molecule of an ABC transporter protein (*e.g.*, an ABC transporter substrate), an ABC transporter antibody, an ABC transporter agonist or antagonist, a peptidomimetic of an ABC transporter agonist or

antagonist, or other small molecule. In one embodiment, the agent stimulates one or more ABC transporter activities. Examples of such stimulatory agents include active ABC transporter protein and a nucleic acid molecule encoding an ABC transporter that has been introduced into the cell. In another embodiment, the agent inhibits one or more ABC transporter activities. Examples of such inhibitory agents include antisense ABC transporter nucleic acid molecules, anti-ABC transporter antibodies, and ABC transporter inhibitors, wherein APP expression is inhibited. These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, the present invention provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant or unwanted expression or activity of an ABC transporter protein or nucleic acid molecule associated with aberrant APP expression. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) ABC transporter expression or activity. In another embodiment, the method involves administering an ABC transporter protein or nucleic acid molecule as therapy to compensate for reduced, aberrant, or unwanted ABC transporter expression or activity.

Stimulation of ABC transporter activity is desirable in situations in which ABC transporter is abnormally downregulated and/or in which increased ABC transporter activity is likely to have a beneficial effect. For example, stimulation of ABC transporter activity is desirable in situations in which an ABC transporter is downregulated and/or in which increased ABC transporter activity is likely to have a beneficial effect. Likewise, inhibition of ABC transporter activity is desirable in situations in which ABC transporter is abnormally upregulated and/or in which decreased ABC transporter activity is likely to have a beneficial effect, such as by reducing the expression of APP in the cell, thereby preventing or alleviating β-amyloid release from the cell and the resulting β-amyloid plaque formation in Alzheimer's disease.

In one embodiment, an agent found to inhibit ABC transporter activity is used in combination with another therapy such that the targeting of that therapy across the blood-brain barrier is achieved.

iii. Pharmacogenomics

The ABC transporter molecules described herein, as well as agents, or modulators which have a stimulatory or inhibitory effect on ABC transporter activity (*e.g.*, ABC transporter gene expression and associated APP expression) as identified by a screening assay described herein can be administered to individuals to treat (prophylactically or therapeutically) ABC-associated disorders associated with aberrant or unwanted ABC transporter activity and associated APP expression. In conjunction with such treatment, pharmacogenomics (*i.e.*, the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically active drug. Thus, a physician or clinician may consider applying knowledge obtained in relevant pharmacogenomics studies in determining whether to administer an ABC transporter molecule or ABC transporter modulator as well as tailoring the dosage and/or therapeutic regimen of treatment with an ABC transporter molecule or ABC transporter modulator.

Information generated from pharmacogenomics approaches can be used to determine appropriate dosage and treatment regimens for prophylactic or therapeutic treatment an individual. This knowledge, when applied to dosing or drug selection, can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with an ABC transporter molecule or ABC transporter modulator, such as a modulator identified by one of the exemplary screening assays described herein.

This invention is further illustrated by the following example which should not be construed as limiting.

EXAMPLES

EXAMPLE 1

TRANSFECTION OF CELLS WITH ABCB9 CONSTRUCTS AND EFFECT ON APP EXPRESSION

293-EBNA (In Vitrogen, Carlsbad, CA) or 293-EBNA cells stably transfected with wild-type human amyloid precursor protein-695 (WT6 cells) were cultured in DMEM supplemented with sodium pyruvate (1 mM) and 10% fetal calf serum. Cells were plated at a density of 100,000 cells per well in 35 mm^2 culture dishes (Falcon™) 18 hours prior to transient transfection experiments. Cultures were then transfected for 48 hours using a Fugene-6 transfection procedure (Boehringer Mannheim, Laval, QC) that entailed the use of 2 μg DNA at a DNA:Fugene-6 ratio of 1:3. Control cultures were transfected with a construct referred to as pExpressβGal which is a vector backbone composed of pcDNA3.1 containing the multiple cloning site of pBluescript into which the LacZ sequence was inserted. Treated cultures were transfected with ABCB9, the polynucleotide and polypeptide sequences of which are disclosed in SEQ ID NOs:1 and 6, respectively or one of two ABCB9 mutant constructs which were subcloned into the pcDNA3.1 vector. Following the 48 hour transfection procedure, cultures were washed once with warm PBS (37°C) and then exposed to DMEM supplemented with sodium pyruvate (1 mM) for 4 hours. After this four hour incubation period, the media was removed, the cultures were washed once with PBS, and were then harvested in 100μl of ice-cold lysis buffer containing 20 mM MOPS (pH 7.2), 5 mM EDTA, 0.01% Nonidet P-40, 75 mM β-glycerol phosphate and a cocktail of protease inhibitors (Boehringer Mannheim, Laval, QC).

To examine intracellular APP levels, cells were harvested with ice-cold lysis buffer and sonicated on ice for 8 seconds using a probe sonicator. Cellular APP levels were quantitated by 10% Tris-Glyine SDS-PAGE Western blot analysis using an anti-APP N-terminal antibody (22C11, Boehringer Mannheim, Laval, QC) (Mills et al., *J. Neurosci. 17*:9415-9422 (1997); Connop et al., *J. Neurochem. 72*:1457-1465 (1999)). Immunoreactive bands were visualized using ECL detection (Amersham, Oakville, ON) and analyzed by standard densitometric techniques.

Statistical significance was determined using an ANOVA with Tukey's *post hoc* analysis. Data are expressed as mean ± SEM with *p<0.05, **p<0.001 *n*=between 3 and 8 for each treatment group.

WT6 cells were transiently transfected with constructs encoding β-galactosidase, ABCB9 or one of two ABCB9 mutant proteins (B9m1, B9m2). After a 48 hour transfection interval, cellular APP levels were quantitated by Western blot analysis. Results demonstrated a statistically significant ($p<0.0001$) increase in the level of APP expression when using wild type ABCB9, compared to both the construct containing β-galactosidase or either of the two mutants. Experiments in which EBNA cells were transiently transfected with constructs encoding β-galactosidase, ABCB9 or one of the two ABCB9 mutant proteins as described above for WT6 cells, demonstrated a statistically significant ($p<0.05$) increase in the level of APP expression when wild type ABCB9 was used when compared to the mutant or control constructs.

EXAMPLE 2

TRANSFECTION OF CELLS WITH ABCG4 CONSTRUCTS AND EFFECT ON APP EXPRESSION

WT-6 and SM4 cells (*i.e.*, 293 EBNA cells stably transfected with a gene encoding either wild type or Swedish mutant Amyloid Precursor Protein -695, respectively) were routinely maintained, seeded on Poly-D-Lysine (Sigma) coated plates, and then transfected as described in Example 1. Control cultures were transfected with β-galactosidase (in the pCEP4 vector) while treated cultures were transfected with ABCG4 (the nucleotide and amino acid sequences of which are disclosed in SEQ ID NOs:4 and 9, respectively or one of 3 ABCG4 mutants altered at the Walker A and/or Walker B motifs (G4mA: K→R, G4mB: D→N, G4mAB: K→R and D→N) subcloned into the pCEP4 vector. After the 48 hour transfection period, the cells were rinsed with 1 ml of warm PBS (37°C). The WT-6 cells were subsequently exposed in 1 ml serum free DMEM supplemented with sodium pyruvate (1mM) for 4 hours, while the SM-4 cells were exposed in serum free DMEM supplemented with sodium pyruvate (1mM) for 16 hours. Transfection efficiency was monitored in each experiment using a β-galactosidase (β-gal) staining kit (In Vitrogen, Carlsbad, CA).

To measure intracellular Amyloid Precursor Protein (APP) levels, the cellular lysate was collected in 100 µl of cold lysis buffer (0.01% Nonidet P-40, 20 mM MOPS, 5 mM

EDTA and 75 mM β-glycerol phosphate, protease inhibitor cocktail (Boehringer Mannheim, Laval, QC)) and sonicated on ice for 8 seconds using a probe sonicator. From each sample, total protein concentration was determined using the bicinchonic acid assay (Pierce, Rockford, Il, USA). Cellular APP levels were quantitated as described in Example 1. Statistical significance was determined using an ANOVA with Tukey's *post hoc* analysis. Data are expressed as mean ± SD with * $p < 0.05$ and ***$p<0.001$ and n =5.

WT6 cells were transiently transfected with constructs encoding β-Galactosidase, ABCG4 or one of three ABCG4 mutant proteins (G4mA, G4mB, G4mAB). After a 48 hr transfection interval cellular APP was quantitated by Western blot analysis. Results demonstrated a statistically significant ($p<0.001$) increase in the level of APP detected in the WT6 cells transfected with wild type ABCG4 when compared to WT6 cells transfected with either the control construct or any one of the mutant ABCG4s.

In a similar set of experiments, SM4 cells were transiently transfected with constructs encoding β-Galactosidase, ABCG4 or one of three ABCG4 mutant proteins. After a 48 hour transfection interval cellular APP was quantitated by Western blot analysis. Results demonstrated a statistically significant ($p<0.05$) increase in the level of APP detected in the WT6 cells transfected with wild type ABCG4 when compared to WT6 cells transfected with either the control construct or any one of the mutant ABCG4s. Data are expressed as mean ± SD with $n = 5$ and statistical significance determined by ANOVA with Tukey's *post hoc* test at *$p<0.05$.

EXAMPLE 3

TRANSFECTION OF CELLS WITH ABCG1 CONSTRUCTS AND EFFECT ON APP EXPRESSION

The present Example describes the modulation in expression of the amyloid precursor protein (APP) and amyloid β peptide production by ABCG1, a member of the ATP-binding cassette transporter super family.

In order to perform these experiments, 293 EBNA cells (InVitrogen, Carlsbad, CA) were stably transfected with Swedish mutant Amyloid Precursor Protein –695 and maintained in DMEM supplemented with sodium pyruvate (1mM) and 10% fetal bovine serum. Eighteen hours prior to transient transfection experiments, the cells were seeded on Poly-D-

Lysine (Sigma) coated 35mm^2 plates at a density of 1.25x10^5 cells/plate. Cultures were then transfected for 48 hours using FuGene-6 (Boehringer Mannheim, Laval, QC) or LipofactAMINE (GIBCO, Burlington, ON) tranfection reagents as per the manufacturer's instructions. Control cultures were transfected with β-galactosidase gene (in the pCEP4 vector), while treated cultures were transfected with ABCG1 (the polynucleotide and amino acid sequences of which are disclosed in SEQ ID NOs:5 and 10 respectively, or one of three ABCG1 mutants altered at the Walker A and/or Walker B motifs (G1mA:K120R, G1mB:D237N, G1mAB:K120R and D237N) subcloned into pCEP4. Walker Motifs are a highly conserved ATP-binding region found in ABC proteins. Following 48 hours in culture, the cells were rinsed with 1ml of warm PBS, followed by culture in serum free DMEM supplemented with sodium pyruvate (1mM) for 16 hours. Transfection efficiency was monitored in each experiment using a β-galactosidase staining kit (Invitrogen, Carlsbad, CA). The protein expression of ABCG1 and the corresponding ABCG1 mutants was confirmed by immunoblot analysis after 48 hours.

To measure intracellular APP levels, cells were harvested with ice-cold lysis buffer and sonicated on ice for 8 seconds using a probe sonicator. For each sample, the total protein concentration was measured using the bicinchonic acid assay (Pierce, Rockford, Il, USA). Cellular proteins were separated by 10% Tris-Glycine SDS-PAGE and analyzed by immunoblot using an anti-APP N-terminal antibody (22C11, Boehringer Mannhein, Laval, QC) (Mills et al., *J. Neuro. 17(24)*:9415-9422, 1997; Connop et al. *J. Neurochem. 72(4)*:1457-1465, 1999) or polyclonal anti-ABCG1 antibody. Immunoreactive bands were visualized using ECL detection (Amersham, Oakville, ON) and analyzed by standard densitometric techniques.

When APP expression was measured in SM4 cells transfected with either ABCG1 or one of the three ABCG1 mutants using FuGene transfection reagents, there was a statistically significant increase in the level of APP expression when using wild type ABCG1 compared to controls. These effects appear to be ABCG1 wild-type mediated since the three inactive mutants (G1mA:K120R, G1mB:D237N, G1mAB:K120R and D237N) used did not mediate the same effect.

When APP expression was measured in SM4 cells transfected with either ABCG1 or one of the three ABCG1 mutants using LiptofectAMINE transfection reagents, there was a statistically significant increase in the level of APP expression when using wild type ABCG1

compared to controls. This increase was slightly reduced when compared to levels seen when using the FuGene transfection reagents. These effects appear to be ABCG1 wild-type mediated since the three inactive mutants (G1mA:K120R, G1mB:D237N, G1mAB:K120R and D237N) used did not mediate the same effect.

Statistical analysis was determined as described in Examples 1 and 2.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.